



Europäisches Patentamt
European Patent Office
Office européen des brevets

⑩ Publication number:

0 255 378
A2

⑫

EUROPEAN PATENT APPLICATION

⑬ Application number: 87306739.1

⑮ Int. Cl.4: C 12 N 15/00

A 01 H 1/00, A 01 H 5/10

// C07K 13/00

⑭ Date of filing: 30.07.87

⑩ Priority: 31.07.86 US 891529 28.07.87 US 78538

⑥ Applicant: CALGENE, INC.
1920 Fifth Street Suite F
Davis California 95616 (US)

⑪ Date of publication of application:
03.02.88 Bulletin 88/05

⑦ Inventor: Kyndi, Jean C.
538 Reed Drive
Davis California 95616 (US)

⑫ Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

Knauf, Vic C.
2454 Elendri Lane
Davis California 95616 (US)

⑬ Representative: Paget, Hugh Charles Edward et al
MEWBURN ELLIS & CO. 2/3 Cunliffe Street
London EC4A 1BQ (GB)

⑭ Seed specific transcriptional regulation.

⑮ Nucleic acid sequences and methods for their use are provided which provide for seed specific transcription, in order to modulate or modify expression in seed, particularly embryo cells. Transcriptional initiation regions are identified and isolated from plant cells and used to prepare expression cassettes which may then be transformed into plant cells for seed specific transcription. The method finds particular use in conjunction with modifying fatty acid production in seed tissue

EP 0 255 378 A2

Description**SEED SPECIFIC TRANSCRIPTIONAL REGULATION****INTRODUCTION**

5

Technical Field

Genetic modification of plant material is provided for seed specific transcription. Production of endogenous products may be modulated or new capabilities provided.

10

Background

The primary emphasis in genetic modification has been directed to prokaryotes and mammalian cells. For a variety of reasons plants have proven more intransigent than other eukaryotic cells in the ability to genetically manipulate the plants. In part, this has been the result of the different goals involved, since for the most part plant modification has been directed to modifying the entire plant or a particular plant part in a live plant, as distinct from modifying cells in culture.

For many applications, it will be desirable to provide for transcription in a particular plant part or at a particular time in the growth cycle of the plant. Toward this end, there is a substantial interest in identifying endogenous plant products whose transcription or expression are regulated in a manner of interest. In identifying such products, one must first look for products which appear at a particular time in the cell growth cycle or in a particular plant part, demonstrate its absence at other times or in other parts, identify nucleic acid sequences associated with the product and then identify the sequence in the genome of the plant in order to obtain the 5'-untranslated sequence associated with transcription. This requires substantial investigation in first identifying the particular sequence, followed by establishing that it is the correct sequence and isolating the desired transcriptional regulatory region. One must then prepare appropriate constructs, followed by demonstration that the constructs are efficacious in the desired manner.

Identifying such sequences is a challenging project, subject to substantial pitfalls and uncertainty. There is, however, substantial interest in being able to genetically modify plants, which justifies the substantial expenditures and efforts in identifying transcriptional sequences and manipulating them to determine their utility.

30

Relevant Literature

Crouch *et al.*, In: *Molecular Form and Function of the Plant Genome*, eds van Vloten-Doting, Groot and Hall, Plenum Publishing Corp. 1985, pp 555-566; Crouch and Sussex, *Planta* (1981) 153:64-74; Crouch *et al.*, *J. Mol. Appl. Genet.* (1983) 2:273-283; and Simon *et al.*, *Plant Molecular Biology* (1985) 5: 191-201, describe various aspects of *Brassica napus* storage proteins. Beachy *et al.*, *EMBO J.* (1985) 4:3047-3053; Sengupta-Gopalan *et al.*, *Proc. Natl. Acad. Sci. USA* (1985) 82:3320-3324; Greenwood and Chrispeels, *Plant Physiol.* (1985) 79:65-71 and Chen *et al.*, *Proc. Natl. Acad. Sci. USA* (1986) 83:8560-8564 describe studies concerned with seed storage proteins and genetic manipulation. Eckes *et al.*, *Mol. Gen. Genet.* (1986) 205:14-22 and Fluhr *et al.*, *Science* (1986) 232:1106-1112 describe the genetic manipulation of light inducible plant genes.

40

SUMMARY OF THE INVENTION

DNA constructs are provided which are employed in manipulating plant cells to provide for seed-specific transcription. Particularly, storage protein transcriptional regions are joined to other than the wild-type gene and introduced into plant genomes to provide for seed-specific transcription. The constructs provide for modulation of endogenous products as well as production of heterologous products.

45

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Novel DNA constructs are provided which allow for modification of transcription in seed, particularly in embryos during seed maturation. The DNA constructs comprise a regulated transcriptional initiation region associated with seed formation, preferably in association with embryogenesis and seed maturation. Of particular interest are those transcriptional initiation regions associated with storage proteins, such as napin, cruciferin, β -conglycinin, phaseolin, or the like. The transcriptional initiation regions may be obtained from any convenient host, particularly plant hosts such as *Brassica*, e.g. *napus* or *campestris*, soybean (*Glycine max*), bean (*Phaseolus vulgaris*), corn (*Zea mays*), cotton (*Gossypium sp.*), safflower (*Carthamus tinctorius*), tomato (*Lycopersicon esculentum*), and *Cuphea* species.

55

Downstream from and under the transcriptional initiation regulation of the seed specific region will be a sequence of interest which will provide for modification of the phenotype of the seed, by modulating the production of an endogenous product, as to amount, relative distribution, or the like, or production of a heterologous expression product to provide for a novel function or product in the seed. The DNA construct will also provide for a termination region, so as to provide an expression cassette into which a gene may be introduced. Conveniently, transcriptional initiation and termination regions may be provided separated in the direction of transcription by a linker or polylinker having one or a plurality of restriction sites for insertion of the gene to be under the transcriptional regulation of the regulatory regions. Usually, the linker will have from 1 to

10, more usually from about 1 to 8, preferably from about 2 to 6 restriction sites. Generally, the linker will be fewer than 100 bp, frequently fewer than 60 bp and generally at least about 5 bp.

The transcriptional initiation region may be native or homologous to the host or foreign or heterologous to the host. By foreign is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced.

Transcriptional initiation regions of particular interest are those associated with the Brassica napus or campestris napin genes, acyl carrier proteins, genes that express from about day 7 to day 40 in seed, particularly having maximum expression from about day 10 to about day 20, where the expressed gene is not found in leaves, while the expressed product is found in seed in high abundance.

The transcriptional cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a sequence of interest, and a transcriptional and translational termination region functional in plants. One or more introns may also be present. The DNA sequence may have any open reading frame encoding a peptide of interest, e.g. an enzyme, or a sequence complementary to a genomic sequence, where the genomic sequence may be an open reading frame, an intron, a non-coding leader sequence, or any other sequence where the complementary sequence will inhibit transcription, messenger RNA processing, e.g. splicing, or translation. The DNA sequence of interest may be synthetic, naturally derived, or combinations thereof. Depending upon the nature of the DNA sequence of interest, it may be desirable to synthesize the sequence with plant preferred codons. The plant preferred codons may be determined from the codons of highest frequency in the proteins expressed in the largest amount in the particular plant species of interest.

In preparing the transcription cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed for joining the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, in vitro mutagenesis, primer repair, restriction, annealing, resection, ligation, or the like may be employed, where insertions, deletions or substitutions, e.g. transitions and transversions, may be involved.

The termination region which is employed will be primarily one of convenience, since the termination regions appear to be relatively interchangeable. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of A. tumefaciens, such as the octopine synthase and nopaline synthase termination regions.

By appropriate manipulations, such as restriction, chewing back or filling in overhangs to provide blunt ends, ligation of linkers, or the like, complementary ends of the fragments can be provided for joining and ligation.

In carrying out the various steps, cloning is employed, so as to amplify the amount of DNA and to allow for analyzing the DNA to ensure that the operations have occurred in a proper manner. A wide variety of cloning vectors are available, where the cloning vector includes a replication system functional in E. coli and a marker which allows for selection of the transformed cells. Illustrative vectors include pBR322, pUC series, M13mp series, PACYC184, etc. Thus, the sequence may be inserted into the vector at an appropriate restriction site(s), the resulting plasmid used to transform the E. coli host, the E. coli grown in an appropriate nutrient medium and the cells harvested and lysed and the plasmid recovered. Analysis may involve sequence analysis, restriction analysis, electrophoresis, or the like. After each manipulation the DNA sequence to be used in the final construct may be restricted and joined to the next sequence, where each of the partial constructs may be cloned in the same or different plasmids.

In addition to the transcription construct, depending upon the manner of introduction of the transcription construct into the plant, other DNA sequences may be required. For example, when using the Ti- or Ri-plasmid for transformation of plant cells, as described below, at least the right border and frequently both the right a left borders of the T-DNA of the Ti-and Ri-plasmids will be joined as flanking regions to the transcription construct. The use of T-DNA for transformation of plant cells has received extensive study and is amply described in EPA Serial No. 120,516, Hoekema, In: The Binary Plant Vector System Offset-drukkerij Kanters B.V., Alblaserdam, 1985, Chapter V, Fraley, et al., Crit. Rev. Plant Sci., 4:1-46, and An et al., EMBO J. (1985) 4:277-284.

Alternatively, to enhance integration into the plant genome, terminal repeats of transposons may be used as borders in conjunction with a transposase. In this situation, expression of the transposase should be inducible, or the transposase inactivated, so that once the transcription construct is integrated into the genome, it should be relatively stably integrated and avoid hopping.

The transcription construct will normally be joined to a marker for selection in plant cells. Conveniently, the marker may be resistance to a biocide, particularly an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, chloramphenicol, or the like. The particular marker employed will be one which will allow for selection of transformed cells as compared to cells lacking the DNA which has been introduced.

A variety of techniques are available for the introduction of DNA into a plant cell host. These techniques include transformation with Ti-DNA employing A. tumefaciens or A. rhizogenes as the transforming agent, protoplast fusion, injection, electroporation, etc. For transformation with Agrobacterium, plasmids can be prepared in E. coli which plasmids contain DNA homologous with the Ti-plasmid, particularly T-DNA. The plasmid may or may not be capable of replication in Agrobacterium, that is, it may or may not have a broad

spectrum prokaryotic replication system, e.g. RK290, depending in part upon whether the transcription construct is to be integrated into the Ti-plasmid or be retained on an independent plasmid. By means of a helper plasmid, the transcription construct may be transferred to the A. tumefaciens and the resulting transformed organism used for transforming plant cells.

5 Conveniently, explants may be cultivated with the A. tumefaciens or A. rhizogenes to allow for transfer of the transcription construct to the plant cells, the plant cells dispersed in an appropriate selective medium for selection, grown to callus, shoots grown and plantlets regenerated from the shoots by growing in rooting medium. The Agrobacterium host will contain a plasmid having the vir genes necessary for transfer of the T-DNA to the plant cells and may or may not have T-DNA. For injection and electroporation, disarmed 10 Ti-plasmids (lacking the tumor genes, particularly the T-DNA region) may be introduced into the plant cell.

10 The constructs may be used in a variety of ways. Particularly, the constructs may be used to modify the fatty acid composition in seeds, that is changing the ratio and/or amounts of the various fatty acids, as to length, unsaturation, or the like. Thus, the fatty acid composition may be varied, enhancing the fatty acids of from 10 to 14 carbon atoms as compared to the fatty acids of from 16 to 18 carbon atoms, increasing or decreasing fatty acids of from 20 to 24 carbon atoms, providing for an enhanced proportion of fatty acids which are saturated or unsaturated, or the like. These results can be achieved by providing for reduction of expression of one or more endogenous products, particularly enzymes or cofactors, by producing a transcription product which is complementary to the transcription product of a native gene, so as to inhibit the maturation and/or expression of the transcription product, or providing for expression of a gene, either endogenous or exogenous, 15 associated with fatty acid synthesis. Expression products associated with fatty acid synthesis include acyl carrier protein, thioesterase, acetyl transacylase, acetyl-coA carboxylase, ketoacyl-synthases, malonyl transacylase, stearoyl-ACP desaturase, and other desaturase enzymes.

20 Alternatively, one may wish to provide various products from other sources including mammals, such as blood factors, lymphokines, colony stimulating factors, interferons, plasminogen activators, enzymes, e.g. superoxide dismutase, chymosin, etc., hormones, rat mammary thioesterase 2, phospholipid acyl desaturases involved in the synthesis of cicosapentaenoic acid, human serum albumin. Another purpose is to increase the 25 level of seed proteins, particularly mutated seed proteins, having an improved amino acid distribution which would be better suited to the nutrient value of the seed. In this situation, one might provide for inhibition of the native seed protein by producing a complementary DNA sequence to the native coding region or non-coding 30 region, where the complementary sequence would not efficiently hybridize to the mutated sequence, or inactivate the native transcriptional capability.

35 The cells which have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.*, Plant Cell Reports (1986) 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, identifying the resulting hybrid having the desired phenotypic characteristic. Two or more generations may be grown to ensure that the subject 40 phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

45 As a host cell, any plant variety may be employed which provides a seed of interest. Thus, for the most part, plants will be chosen where the seed is produced in high amounts or a seed specific product of interest is involved. Seeds of interest include the oil seeds, such as the Brassica seeds, cotton seeds, soybean, safflower, sunflower, or the like; grain seeds, e.g. wheat, barley, rice, clover, corn, or the like.

50 Identifying useful transcriptional initiation regions may be achieved in a number of ways. Where the seed protein has been or is isolated, it may be partially sequenced, so that a probe may be designed for identifying messenger RNA specific for seed. To further enhance the concentration of the messenger RNA specifically 55 associated with seed, cDNA may be prepared and the cDNA subtracted with messenger RNA or cDNA from non-seed associated cells. The residual cDNA may then be used for probing the genome for complementary sequences, using an appropriate library prepared from plant cells. Sequences which hybridize to the cDNA 60 may then be isolated, manipulated, and the 5'-untranslated region associated with the coding region isolated and used in expression constructs to identify the transcriptional activity of the 5'-untranslated region.

55 In some instances, the research effort may be further shortened by employing a probe directly for screening a genomic library and identifying sequences which hybridize to the probe. The sequences will be manipulated as described above to identify 5'-untranslated region.

60 The expression constructs which are prepared employing the 5'-untranslated regions may be transformed into plant cells as described previously for determination of their ability to function with a heterologous structural gene (other than the wild-type open reading frame associated with the 5'-untranslated region) and the seed specificity. In this manner, specific sequences may be identified for use with sequences for seed specific transcription. Expression cassettes of particular interest include transcriptional initiation regions from napin genes, particularly Brassica napin genes, more particularly Brassica napus or Brassica campestris genes, regulating structural genes associated with lipid production, particularly fatty acid production, including acyl carrier proteins, which may be endogenous or exogenous to the particular plant, such as spinach acyl carrier protein, Brassica acyl carrier protein, acyl carrier protein, either napus or campestris, Cuphea acyl carrier protein, acetyl transacylase, malonyl transacylase, β -ketoacyl synthases I and II, thioesterase, particularly thio esterase II, from plant, mammalian, or bacterial sources, for example rat thioesterase II, acyl ACP, or phospholipid acyl desaturases.

65 The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTALMaterials and MethodsCloning Vectors

Cloning vectors used include the pUC vectors, pUC8 and pUC9 (Vieira and Messing, *Gene* (1982) 19:259-268); pUC18 and pUC19 (Norraander et al., *Gene* (1983) 26:101-106; Yanisch-Perron et al., *Gene* (1985) 33:103-119), and analogous vectors exchanging chloramphenicol resistance (CAM) as a marker for the ampicillin resistance of the pUC plasmids described above (pUC-CAM [pUC12-Cm, pUC13-Cm] Buckley, D., Ph.D. Thesis, U.C.S.D., CA 1985). The multiple cloning sites of pUC18 and pUC19 vectors were exchanged with those of pUC-CAM to create pCGN565 and pCGN566 which are CAM resistant. Also used were pUC118 and pUC119, which are respectively, pUC18 and pUC19 with the intergenic region of M13, from an HgiAI site at 5465 to the AhaII site at 5941, inserted at the NdeI site of pUC. (Available from Vieira J. and Messing, J. Waksman Institute, Rutgers University, Rutgers, N.J.)

5

10

15

Materials

Terminal deoxynucleotide transferase (TDT), RNaseH, *E. coli* DNA polymerase, T4 kinase, and restriction enzymes were obtained from Bethesda Research Laboratories; *E. coli* DNA ligase was obtained from New England Biolabs; reverse transcriptase was obtained from Life Sciences, Inc.; isotopes were obtained from Amersham; X-gal was obtained from Bachem, Inc. Torrance, CA.

20

25

Example 1Construction of a Napin Promoter

There are 298 nucleotides upstream of the ATG start codon of the napin gene on the pgN1 clone, a 3.3 kb EcoRI fragment of *B. napus* genomic DNA containing a napin gene cloned into pUC8 (available from Marti Crouch, University of Indiana). pgN1 DNA was digested with EcoRI and SstI and ligated to EcoRI/SstI digested pCGN706. (pCGN706 is an XbaI/PstI fragment containing 3' and polyadenylation sequences of another napin cDNA clone pN2 (Crouch et al., 1983 *supra*) cloned in pCGN566 at the Sall and PstI sites.) The resulting clone pCGN707 was digested with Sall and treated with the enzyme Bal31 to remove some of the coding region of the napin gene. The resulting resected DNA was digested with SmaI after the Bal31 treatment and religated. One of the clones, pCGN713, selected by size, was subcloned by EcoRI and BamHI digestion into both EcoRI/BamHI digested pEMBL18 (Dente et al., *Nucleic Acids Res.* (1983) 11:1645-1655) and pUC118 to give E418 and E4118 respectively. The extent of Bal31 digestion was confirmed by Sanger dideoxy sequencing of E418 template. The Bal31 deletion of the promoter region extended only to 57 nucleotides downstream of the start codon, thus containing the 5' end of the napin coding sequence and about 300 bp of the 5' non-coding region. E4118 was tailored to delete all of the coding region of napin including the ATG start codon by *in vitro* mutagenesis by the method of Zoller and Smith (*Nucleic Acids Res.* (1982) 10:6487-6500) using an oligonucleotide primer 5'-GATGTTTGTATGTGGGCCCTAGGAGATC-3'. Screening for the appropriate mutant was done by two transformations into *E. coli* strain JM83 (Messing J., In: *Recombinant DNA Technical Bulletin*, NIH Publication No. 79-99, 2 No. 2, 1979, pp 43-48) and SmaI digestion of putative transformants. The resulting napin promoter clone is pCGN778 and contains 298 nucleotides from the EcoRI site of pgN1 to the A nucleotide just before the ATG start codon of napin. The promoter region was subcloned into a chloramphenicol resistant background by digestion with EcoRI and BamHI and ligation to EcoRI/BamHI digested pCGN565 to give pCGN779c.

30

35

40

45

50

55

Extension of the Napin Promoter Clone

pCGN779c contains only 298 nucleotides of potential 5'-regulatory sequence. The napin promoter was extended with a 1.8 kb fragment found upstream of the 5'-EcoRI site on the original λ BnNa clone. The ~3.5 kb XbaI fragment of λ BnNa (available from M. Crouch), which includes the napin region, was subcloned into Sall-digested pUC119 to give pCGN930. A HindIII site close to a 5' XbaI site was used to subclone the HindIII/EcoRI fragment of pCGN930 into HindIII/EcoRI digested Bluescript + (Vector Cloning Systems, San Diego, CA) to give pCGN942. An extended napin promoter was made by ligating pCGN779c digested with EcoRI and PstI and pCGN942 digested with EcoRI and PstI to make pCGN943. This promoter contains ~2.1 kb of sequence upstream of the original ATG of the napin gene contained on λ BnNa. A partial sequence of the promoter region is shown in Figure 1.

Napin Cassettes

The extended napin promoter and a napin 3'-regulatory region is combined to make a napin cassette for expressing genes seed-specifically. The napin 3'-region used is from the plasmid pCGN1924 containing the XbaI/EcoRI fragment from pgN1 (XbaI site is located 18 nucleotides from the stop codon of the napin gene) subcloned into EcoRI/Sall digested pCGN565. HindIII/PstI digested pCGN943 and pCGN1924 are ligated to make the napin cassette pCGN744, with unique cloning sites SmaI, Sall, and PstI for inserting genes.

60

65

Construction of cDNA Library from Spinach Leaves

5 Total RNA was extracted from young spinach leaves in 4M guanidine thiocyanate buffer as described by Facciotti et al. (Biotechnology (1985) 3:241-246). Total RNA was subjected to oligo(dT)-cellulose column chromatography two times to yield poly(A) + RNA as described by Maniatis et al., (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York. A cDNA library was constructed in pUC13-Cm according to the method of Gubler and Hoffman, (Gene (1983) 25:263-269) with slight modifications. RNasin was omitted in the synthesis of first strand cDNA as it interfered with second strand synthesis if not completely removed, and dCTP was used to tail the vector DNA and dGTP to tail double-stranded cDNA instead of the reverse as described in the paper. The annealed cDNA was transformed to competent *E. coli* JM83 (Messing 10 (1979) *supra*) cells according to Hanahan (*J. Mol. Biol.* (1983) 166:557-580) and spread onto LB agar plates (Miller (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) containing 50 µg/ml chloramphenicol and 0.005% X-Gal.

Identification of Spinach ACP-I cDNA

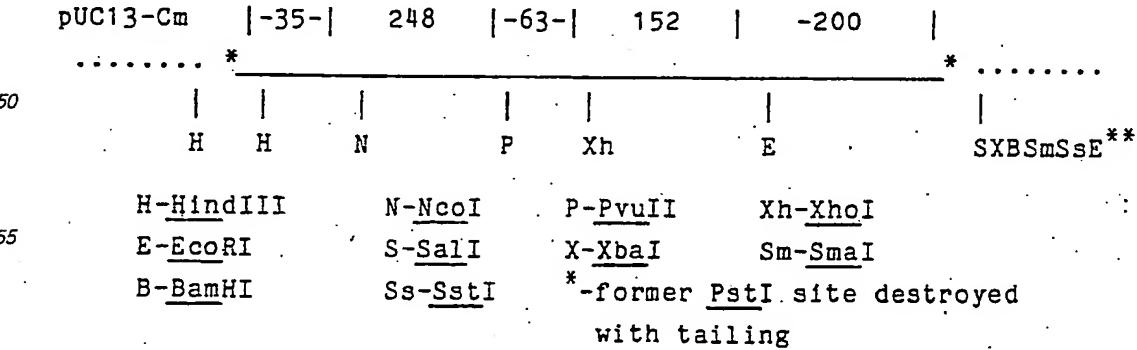
15 A total of approximately 8000 cDNA clones were screened by performing Southern blots (Southern, J. Mol. Biol. (1975) 98:503) and dot blot (described below) hybridizations with clone analysis DNA from 40 pools representing 200 cDNA clones each (see below). A 5' end labeled synthetic oligonucleotide (ACPP4) that is at least 66% homologous with a 16 amino acid region of spinach ACP-I (5'-GATGTCTTGGCCTTGCCTCATC-
 20 CACATTGATACCAACTCCTCCTC-3') is the complement to a DNA sequence that could encode the 16 amino acid peptide glu-glu-glu-phe-gly-ile-asn-val-asp-glu-asp-lys-ala-gln-asp-ile, residues 49-64 of spinach ACP-I (Kuo and Ohlrogge, Arch. Biochem. Biophys. (1984) 234:290-296) and was used for an ACP probe.

25 Clone analysis DNA for Southern and dot blot hybridizations was prepared as follows. Transformants were transferred from agar plates to LB containing 50 µg/ml chloramphenicol in groups of ten clones per 10 ml media. Cultures were incubated overnight in a 37°C shaking incubator and then diluted with an equal volume of media and allowed to grow for 5 more hours. Pools of 200 cDNA clones each were obtained by mixing contents of 20 samples. DNA was extracted from these cells as described by Birnboim and Doly (Nucleic Acids Res. (1979) 7:1513-1523). DNA was purified to enable digestion with restriction enzymes by extractions with phenol and chloroform followed by ethanol precipitation. DNA was resuspended in sterile, distilled water and 1 µg of each of the 40 pooled DNA samples was digested with EcoRI and HindIII and electrophoresed through 0.7% agarose gels. DNA was transferred to nitrocellulose filters following the blot hybridization technique of Southern.

ACPP4 was 5' end-labeled using γ -³²P dATP and T4 kinase according to the manufacturer's specifications. Nitrocellulose filters from Southern blot transfer of clone analysis DNA were hybridized (24 hours, 42°C) and washed according to Berent *et al.* (*BioTechniques* (1985) 3:208-220). Dot blots of the same set of DNA pools were prepared by applying 1 μ g of each DNA pool to nylon membrane filters in 0.5 M NaOH. These blots were hybridized with the probe for 24 hours at 42°C in 50% formamide/1% SDS/1 M NaCl, and washed at room temperature in 2X SSC/0.1% SDS (1X SSC = 0.15M NaCl; 0.015M Na citrate; SDS-sodium dodecylsulfate). DNA from the pool which was hybridized by the ACPP4 oligoprobe was transformed to JM83 cells and plated as above to yield individual transformants. Dot blots of these individual cDNA clones were prepared by applying DNA to nitrocellulose filters which were hybridized with the ACPP4 oligonucleotide probe and analyzed using the same conditions as for the Southern blots of pooled DNA samples.

Nucleotide Sequence Analysis

45 The positive clone, pCGNTSOL, was analyzed by digestion with restriction enzymes and the following partial map was obtained.



** polylinker with available restriction sites indicated

The cDNA clone was subcloned into pUC118 and pUC119 using standard laboratory techniques of restriction, ligation, transformation, and analysis (Maniatis et al., (1982) *supra*). Single-stranded DNA template was prepared and DNA sequence was determined using the Sanger dideoxy technique (Sanger et al. (1977)

Proc. Nat. Acad. Sci. USA 74:5463-5467). Sequence analysis was performed using a software package from Intelli-Genetics, Inc.

pCGN1SOL contains an (approximately) 700 bp cDNA insert including a stretch of A residues at the 3' terminus which represents the poly(A) tail of the mRNA. An ATG codon at position 61 is presumed to encode the MET translation initiation codon. This codon is the start of a 411 nucleotide open reading frame, of which, nucleotides 229-471 encode a protein whose amino acid sequence corresponds almost perfectly with the published amino acid sequence of ACP-I of Kuo and Ohlrogge *supra* as described previously. In addition to mature protein, the pCGN1SOL also encodes a 56 residue transit peptide sequence, as might be expected for a nuclear-encoded chloroplast protein.

Napin - ACP Construct

pCGN796 was constructed by ligating pCGN1SOL digested with HindIII/BamHI, pUC8 digested with HindIII and BamHI and pUC118 digested with BamHI. The ACP gene from pCGN796 was transferred into a chloramphenicol background by digestion with BamHI and ligation with BamHI digested pCGN565. The resulting pCGN1902 was digested with EcoRI and SmaI and ligated to EcoRI/SmaI digested pUC118 to give pCGN1920. The ACP gene in pCGN1920 was digested at the NcoI site, filled in by treatment with the Klenow fragment, digested with SmaI and religated to form pCGN1919. This eliminated the 5'-coding sequences from the ACP gene and regenerated the ATG. This ACP gene was flanked with PstI sites by digesting pCGN1919 with EcoRI, filling in the site with the Klenow fragment and ligating a PstI linker. This clone is called pCGN945.

The ACP gene of pCGN945 was moved as a BamHI/PstI fragment to pUC118 digested with BamHI and PstI to create pCGN945a so that a SmaI site (provided by the pUC118) would be at the 5'-end of the ACP sequences to facilitate cloning into the napin cassette pCGN944. pCGN945a digested with SmaI and PstI was ligated to pCGN944 digested with SmaI and PstI to produce the napin ACP cassette pCGN946. The napin ACP cassette was then transferred into the binary vector pCGN783 by cloning from the HindIII site to produce pCGN948.

Construction of the Binary Vector pCGN783

pCGN783 is a binary plasmid containing the left and right T-DNA borders of *A. tumefaciens* (Barker et al., *Plant Mol. Biol.* (1983) 2:335-350); the gentamicin resistance gene of pPH1J1 (Hirsch et al., *Plasmid* (1984), 12:139-141) the 35S promoter of cauliflower mosaic virus (CaMV) (Gardner et al., *Nucleic Acids Res.* (1981) 9:2871-2890), the kanamycin resistance gene of Tn5 (Jorgenson et al., *infra* and Wolff et al., *Ibid* (1985) 13:355-367) and the 3' region from transcript 7 of pTiA6 (Barker et al., *supra* (1983)).

To obtain the gentamicin resistance marker, the gentamicin resistance gene was isolated from a 3.1 kb EcoRI-PstI fragment of pPH1J1 and cloned into pUC9 yielding pCGN549. The HindIII-BamHI fragment containing the gentamicin resistance gene was substituted for the HindIII-BglII fragment of pCGN587 creating pCGN594.

pCGN587 was prepared as follows: The HindIII-SmaI fragment of Tn5 containing the entire structural gene for APHII (Jorgenson et al., *Mol. Gen. Genet.* (1979) 177:65) was cloned into pUC8 (Vieira and Messing, *Gene* (1982) 19:259), converting the fragment into a HindIII-EcoRI fragment, since there is an EcoRI-site immediately adjacent to the SmaI site. The PstI-EcoRI fragment containing the 3'-portion of the APHII gene was then combined with an EcoRI-BamHI-SmaI-PstI linker into the EcoRI site of pUC7 (pCGN546W). Since this construct does not confer kanamycin resistance, kanamycin resistance was obtained by inserting the BglII-PstI fragment of the APHII gene into the BamHI-PstI site (pCGN546X). This procedure reassembles the APHII gene, so that Eco sites flank the gene. An ATG codon was upstream from and out of reading frame with the ATG initiation codon of APHII. The undesired ATG was avoided by inserting a Sau3A-PstI fragment from the 5'-end of APHII, which fragment lacks the superfluous ATG, into the BamHI-PstI site of pCGN546W to provide plasmid pCGN550.

The EcoRI fragment containing the APHII gene was then cloned into the unique EcoRI site of pCGN451, which contains an octopine synthase cassette for expression, to provide pCGN552 (1ATG).

pCGN451 includes an octopine cassette which contains about 1556 bp of the 5' non-coding region fused via an EcoRI linker to the 3' non-coding region of the octopine synthase gene of pTiA6. The pTi coordinates are 11,207 to 12,823 for the 3' region and 13,643 to 15,208 for the 5' region as defined by Barker et al., *Plant Mol. Biol.* (1983) 2:325.

The 5' fragment was obtained as follows. A small subcloned fragment containing the 5' end of the coding region, as a BamHI-EcoRI fragment was cloned in pBR322 as plasmid pCGN407. The BamHI-EcoRI fragment has an XbaI site in the coding region, while pBR322 has two XbaI sites. pCGN407 was digested with XbaI, resected with Bal31 nuclease and EcoRI linkers added to the fragments. After EcoRI and BamHI digestion, the fragments were size fractionated, the fractions cloned and sequenced. In one case, the entire coding region and 10 bp of the 5' non-translated sequences had been removed leaving the 5' non-translated region, the mRNA cap site and 16 bp of the 5' non-translated region (to a BamHI site) intact. This small fragment was obtained by size fractionation on a 7% acrylamide gel and fragments approximately 130 bp long eluted.

This size fractionated DNA was ligated into M13mp9 and several clones sequenced and the sequence compared to the known sequence of the octopine synthase gene. The M13 construct was designated p14, which plasmid was digested with BamHI and EcoRI to provide the small fragment which was ligated to a XbaI fragment containing upstream 5' sequences from pTiA6 (Garfinkel and Nester, *J. Bacteriol.* (1980)

5

10

15

20

25

30

35

40

45

50

55

60

65

144:732) and to an EcoRI to Xhol fragment containing the 3' sequences.

The resulting Xhol fragment was cloned into the Xhol site of a pUC8 derivative, designated pCGN426. This plasmid differs from pUC8 by having the sole EcoRI site filled in with DNA polymerase I, and having lost the PstI and HindIII site by nuclease contamination of HincII restriction endonuclease, when a Xhol linker was inserted into the unique HincII site of pUC8. The resulting plasmid pCGN451 has a single EcoRI site for the insertion of protein coding sequences between the 5' non-coding region (which contains 1,550 bp of 5' non-transcribed sequence including the right border of the T-DNA, the mRNA cap site and 16 bp of 5' non-translated sequence) and the 3' region (which contains 267 bp of the coding region; the stop codon, 196 bp of 3' non-translated DNA, the polyA site and 1,153 bp of 3' non-transcribed sequence). pCGN451 also provides the right T-DNA border.

10 The resulting plasmid pCGN451 having the ocs 5' and the ocs 3' in the proper orientation was digested with EcoRI and the EcoRI fragment from pCGN551 containing the intact kanamycin resistance gene inserted into the EcoRI site to provide pCGN552 having the kanamycin resistance gene in the proper orientation.

This ocs/KAN gene was used to provide a selectable marker for the trans type binary vector pCGN587.

15 The 5' portion of the engineered octopine synthase promoter cassette consists of pTiA6 DNA from the Xhol at bp 15208-13644 (Barker's numbering), which also contains the T-DNA boundary sequence (border) implicated in T-DNA transfer. In the plasmid pCGN587, the ocs/KAN gene from pCGN552 provides a selectable marker as well as the right border. The left boundary region was first cloned in M13mp9 as a HindIII-SmaI piece (pCGN502) (base pairs 602-2213) and recloned as a KpnI-EcoRI fragment in pCGN565 to provide pCGN580. pCGN565 is a cloning vector based on pUC8-Cm, but containing pUC18 linkers. pCGN580 was linearized with BamHI and used to replace the smaller BglII fragment of pVCK102 (Knauf and Nester, Plasmid (1982) 8:45), creating pCGN585. By replacing the smaller Sall fragment of pCGN585 with the Xhol fragment from pCGN552 containing the ocs/KAN gene, pCGN587 was obtained.

20 The pCGN594 HindIII-BamHI region, which contains an 5'-ocs-kanamycin-ocs-3' (ocs is octopine synthase with 5' designating the promoter region and 3' the terminator region, see U.S. application serial no. 775,923, filed September 13, 1985) fragment was replaced with the HindIII-BamHI polylinker region from pUC18.

pCGN566 contains the EcoRI-HindIII linker of pUC18 inserted into the EcoRI-HindIII sites of pUC13-Cm. The HindIII-BglII fragment of pNW31C-8,29-1 (Thomashow et al., Cell (1980) 19:729) containing ORF1 and -2 of pTiA6 was subcloned into the HindIII-BamHI sites of pCGN566 producing pCGN703.

25 The Sau3A fragment of pCGN703 containing the 3' region of transcript 7 (corresponding to bases 2396-2920 of pTiA6 (Barker et al., (1983) supra) was subcloned into the BamHI site of pUC18 producing pCGN709. The EcoRI-SmaI polylinker region of pCGN709 was substituted with the EcoRI-SmaI fragment of pCGN587, which contains the kanamycin resistance gene (APH3-II) producing pCGN726.

30 The EcoRI-Sall fragment of pCGN726 plus the BglII-EcoRI fragment of pCGN734 were inserted into the BamHI-Sall site of pUC8-Cm producing pCGN738. pCGN726c is derived from pCGN738 by deleting the 900 bp EcoRI-EcoRI fragment.

35 To construct pCGN167, the Alul fragment of CaMV (bp 7144-7735) (Gardner et al., Nucl. Acid Res. (1981) 9:2871-2888) was obtained by digestion with Alul and cloned into the HincII site of M13mp7 (Messing et al., Nucl. Acids Res. (1981) 9:309-321) to create C614. An EcoRI digest of C614 produced the EcoRI fragment from C614 containing the 35S promoter which was cloned into the EcoRI site of pUC8 (Vieira and Messing, Gene (1982) 19:259) to produce pCGN146.

40 To trim the promoter region, the BglII site (bp 7670) was treated with BglII and resected with Bal31 and subsequently a BglII linker was attached to the Bal31 treated DNA to produce pCGN147.

45 pCGN148a containing a promoter region, selectable marker (KAN with 2 ATG's) and 3' region, was prepared by digesting pCGN528 with BglII and inserting the BamHI-BglII promoter fragment from pCGN147. This fragment was cloned into the BglII site of pCGN528 so that the BglII site was proximal to the kanamycin gene of pCGN528.

50 The shuttle vector used for this construct, pCGN528, was made as follows. pCGN525 was made by digesting a plasmid containing Tn5 which harbors a kanamycin gene (Jorgenson et al., Mol. Gen. Genet. 1979) 177:65 with HindIII-BamHI and inserting the HindIII-BamHI fragment containing the kanamycin gene into the HindIII-BamHI sites in the tetracycline gene of pACYC184 (Chang and Cohen, J. Bacteriol. (1978) 134:1141-1156); pCGN526 was made by inserting the BamHI fragment 19 of pTiA6 (Thomashow et al., Cell (1980) 19:729-739), modified with Xhol linkers inserted into the SmaI site, into the BamHI site of pCGN525. pCGN528 was obtained by deleting the small Xhol fragment from pCGN526 by digesting with Xhol and religating.

55 pCGN149a was made by cloning the BamHI-kanamycin gene fragment from pMB9KanXXI into the BamHI site of pCGN148a.

pMB9KanXXI is a pUC4K variant (Vieira and Messing, Gene (1982) 19:259-268) which has the Xhol site missing but contains a functional kanamycin gene from Tn903 to allow for efficient selection in Agrobacterium.

60 pCGN149a was digested with BglII and SphI. This small BglII-SphI fragment of pCGN149a was replaced with the BamHI-SphI fragment from M1 (see below) isolated by digestion with BamHI and SphI. This produces pCGN167, a construct containing a full length CaMV promoter, 1ATG-kanamycin gene, 3' end and the bacterial Tn903-type kanamycin gene. M1 is an EcoRI fragment from pCGN546X (see construction of pCGN587) and was cloned into the EcoRI cloning site of M13mp9 in such a way that the PstI site in the 1ATG-kanamycin gene was proximal to the polylinker region of M13mp9.

The HindIII-BamHI fragment in the pCGN167 containing the CaMV-35S promoter, 1ATG-kanamycin gene and the BamHI-fragment 19 of pTiA6 was cloned into the BamHI-HindIII sites of pUC19 creating pCGN976. The 35S promoter and 3' region from transcript 7 was developed by inserting a 0.7 kb HindIII-EcoRI fragment of pCGN976 (35S promoter) and the 0.5 kb EcoRI-SalI fragment of pCGN709 (transcript 7:3') into the HindIII-SalI sites of pCGN566 creating pCGN766c.

The 0.7 kb HindIII-EcoRI fragment of pCGN766c (CaMV-35S promoter) was ligated to the 1.5 kb EcoRI-SalI fragment in pCGN726c (1ATG-KAN 3' region) followed by insertion into the HindIII-SalI sites of pUC119 to produce pCGN778. The 2.2 kb region of pCGN778, HindIII-SalI fragment containing the CaMV-35S promoter and 1ATG-KAN-3' region was used to replace the HindIII-SalI linker region of pCGN739 to produce pCGN783.

Transfer of the Binary Vector pCGN948 into Agrobacterium

pCGN948 was introduced into Agrobacterium tumefaciens EHA101 (Hood et al., J. Bacteriol. (1986) 168:1291-1301) by transformation. An overnight 2 ml culture of EHA101 was grown in MG/L broth at 30°C. 0.5 ml was inoculated into 100 ml of MG/L broth (Garfinkel and Nester, J. Bacteriol. (1980) 144:732-743) and grown in a shaking incubator for 5 h at 30°C. The cells were pelleted by centrifugation at 7K, resuspended in 1 ml of MG/L broth and placed on ice. Approximately, 1 µg of pCGN948 DNA was placed in 100 µl of MG/L broth to which 200 µl of the EHA101 suspension was added; the tube containing the DNA-cell mix was immediately placed into a dry ice/ethanol bath for 5 minutes. The tube was quick thawed by 5 minutes in 37°C water bath followed by 2 h of shaking at 30°C after adding 1 ml of fresh MG/L medium. The cells were pelleted and spread onto MG/L plates (1.5% agar) containing 100 mg/l gentamicin. Plasmid DNA was isolated from individual gentamicin-resistant colonies, transformed back into E. coli, and characterized by restriction enzyme analysis to verify that the gentamicin-resistant EHA101 contained intact copies of pCGN948. Single colonies are picked and purified by two more streakings on MG/L plates containing 100 mg/l gentamicin.

Transformation and Regeneration of B. Napus

Seeds of Brassica napus cv Westar were soaked in 95% ethanol for 4 minutes. They were sterilized in 1% solution of sodium hypochlorite with 50 µl of "Tween 20" surfactant per 100 ml sterilant solution. After soaking for 45 minutes, seeds were rinsed 4 times with sterile distilled water. They were planted in sterile plastic boxes 7 cm wide, 7 cm long, and 10 cm high (Magenta) containing 50 ml of 1/10th concentration of MS (Murashige minimal organics medium, Gibco) with added pyridoxine (500 µg/l), nicotinic acid (50 µg/l), glycine (200 µg/l) and solidified with 0.6% agar. The seeds germinated and were grown at 22°C in a 16h-8h light-dark cycle with light intensity approximately 65 µEm⁻²s⁻¹. After 5 days the seedlings were taken under sterile conditions and the hypocotyls excised and cut into pieces of about 4 mm in length. The hypocotyl segments were placed on a feeder plate or without the feeder layer on top of a filter paper on the solidified B5 0/1/1 or B5 0/1/0 medium. B5 0/1/0 medium contains B5 salts and vitamins (Gamborg, Miller and Ollma, Experimental Cell Res. (1968) 50:151-158), 3% sucrose, 2,4-dichlorophenoxyacetic acid (1.0 mg/l), pH adjusted to 5.8, and the medium is solidified with 0.6% Phytagar; B5 0/1/1 is the same with the addition of 1.0 mg/l kinetin. Feeder plates were prepared 24 hours in advance by pipetting 1.0 ml of a stationary phase tobacco suspension culture (maintained as described in Fillatti et al., Molecular General Genetics (1987) 206:192-199) onto B5 0/1/0 or B5 0/1/1 medium. Hypocotyl segments were cut and placed on feeder plates 24 hours prior to Agrobacterium treatment.

Agrobacterium tumefaciens (strain EHA101 x 948) was prepared by incubating a single colony of Agrobacterium in MG/L broth at 30°C. Bacteria were harvested 16 hours later and dilutions of 10⁸ bacteria per ml were prepared in MG/L broth. Hypocotyl segments were inoculated with bacteria by placing the segments in an Agrobacterium suspension and allowing them to sit for 30-60 minutes, then removing and transferring to Petri plates containing B5 0/1/1 or 0/1/0 medium (0/1/1 intends 1 mg/l 2,4-D and 1 mg/l kinetin and 0/1/0 intends no kinetin). The plates were incubated in low light at 22°C. The co-incubation of bacteria with the hypocotyl segments took place for 24-48 hours. The hypocotyl segments were removed and placed on B5 0/1/1 or 0/1/0 containing 500 mg/l carbenicillin (kanamycin sulfate at 10, 25, or 50 mg/l was sometimes added at this time) for 7 days in continuous light (approximately 65 µEm⁻²S⁻¹) at 22°C. The segments were transferred to B5 salts medium containing 1% sucrose, 3 mg/l benzylamino purine (BAP) and 1 mg/l zeatin. This was supplemented with 500 mg/l carbenicillin, 10, 25, or 50 mg/l kanamycin sulfate, and solidified with 0.6% Phytagar (Gibco). Thereafter, explants were transferred to fresh medium every two weeks.

After one month green shoots developed from green calli which were selected on media containing kanamycin. Shoots continued to develop for three months. The shoots were cut from the calli when they were at least 1 cm high and placed on B5 medium with 1% sucrose, no added growth substances, 300 mg/l carbencillin, and solidified with 0.6% phytagar. The shoots continued to grow and several leaves were removed to test for neomycin phosphotransferase II (NPTII) activity. Shoots which were positive for NPTII activity were placed in Magenta boxes containing B5 0/1/1 medium with 1% sucrose, 2 mg/l indolebutyric acid, 200 mg/l carbenicillin, and solidified with 0.6% Phytagar. After a few weeks the shoots developed roots and were transferred to soil. The plants were grown in a growth chamber at 22°C in a 16-8 hours light-dark cycle with light intensity 220 µEm⁻²s⁻¹ and after several weeks were transferred to the greenhouse.

Southern Data

Regenerated *B. napus* plants from cocultivations of *Agrobacterium tumefaciens* EHA101 containing pCGN948 and *B. napus* hypocotyls were examined for proper integration and embryo-specific expression of the spinach leaf ACP gene. Southern analysis was performed using DNA isolated from leaves of regenerated

5 plants by the method of Dellaporta *et al.*, (Plant Mol. Biol. Rep. (1983) 1:19-21) and purified once by banding in CsCl. DNA (10 µg) was digested with the restriction enzyme EcoRI, electrophoresed on a 0.7% agarose gel and blotted to nitrocellulose (see Maniatis *et al.*, (1982) *supra*). Blots were probed with pCGN945 DNA containing 1.8 kb of the spinach ACP sequence or with the EcoRI/ HindIII fragment isolated from pCGN936c (made by transferring the HindIII/EcoRI fragment of pCGN930 into pCGN566) containing the napin 5' sequences labeled with ³²P-dCTP by nick translation (described by the manufacturer, BRL Nick Translation Reagent Kit, Bethesda Research Laboratories, Gaithersburg, MD). Blots were prehybridized and hybridized in 50% formamide, 10x Denhardt's 5xSSC, 0.1% SDS, 5 mM EDTA, 100 µg/ml calf thymus DNA and 10% dextran sulfate (hybridization only) at 42°C. (Reagents described in Maniatis *et al.*, (1982) *supra*.) Washes were in 1xSSC, 0.1% SDS, 30 min and twice in 0.1xSSC, 0.1% SDS at 55°C.

10 15 Autoradiograms showed two bands of approximately 3.3 and 3.2 kb hybridized in the EcoRI digests of DNA from four plants when probed with the ACP gene (pCGN945) indicating proper integration of the spinach leaf ACP construct in the plant genome since 3.3 and 3.2 kb EcoRI fragments are present in the T-DNA region of pCGN948. The gene construct was present in single or multiple loci in the different plants as judged by the number of plant DNA-construct DNA border fragments detected when probed with the napin 5' sequences.

20

Northern Data

25 Expression of the integrated spinach leaf ACP gene from the napin promoter was detected by Northern analysis in seeds but not leaves of one of the transformed plants shown to contain the construct DNA. Developing seeds were collected from the transformed plant 21 days post-anthesis. Embryos were dissected from the seeds and frozen in liquid nitrogen. Total RNA was isolated from the seed embryos and from leaves of the transformed plant by the method of Crouch *et al.*, Virology (1985) 140:281-288) and blotted to nitrocellulose (Thomas, Proc. Natl. Acad. Sci. USA (1980) 77:5201-5205). Blots were prehybridized, hybridized, and washed as described above. The probe was an isolated PstI/BamHI fragment from pCGN945 containing only spinach leaf ACP sequences labeled by nick translation.

30 An RNA band of ~0.8 kb was detected in embryos but not leaves of the transformed plant indicating seed-specific expression of the spinach leaf ACP gene.

Example II35 Construction of *B. campestris* Napin Promoter Cassette

A BglII partial genomic library of *B. campestris* DNA was made in the lambda vector Charon 35 using established protocols (Maniatis *et al.*, 1982, *supra*). The titer of the amplified library was ~ 1.2 x 10⁹ phage/ml. Four hundred thousand recombinant bacteriophage were plated at a density of 10⁵ per 9 x 9 in. NZY plate (NZYM as described in Maniatis *et al.*, 1982, *supra*) in NZY + 10 mM MgSO₄ + 0.9% agarose after adsorption to DH1 *E. coli* cells (Hanahan, Mol. Biol. (1983) 166:557) for 20 min at 37°C. Plates were incubated at 37°C for ~ 13 hours, cooled at 4°C for 2.5 hours and the phage were lifted onto Gene Screen Plus (New England Nuclear) by laying precut filters over the plates for approximately 1 min and peeling them off. The adsorbed phage DNA was immobilized by floating the filter on 1.5 M NaCl, 0.5 M NaOH for 1 min., neutralizing in 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0 for 2 min. and 2XSSC for 3 min. Filters were air dried until just damp, prehybridized and hybridized at 42°C as described for Southern analysis. Filters were probed for napin-containing clones using an XbaI/SalI fragment of the cDNA clone BE5 which was isolated from the *B. campestris* seed cDNA library described using the probe pN1 (Crouch *et al.*, 1983, *supra*). Three plaques were hybridized strongly on duplicate filters and were plaque purified as described (Maniatis *et al.*, 1982, *supra*).

40 45 50 One of the clones named lambda CGN1-2 was restriction mapped and the napin gene was localized to overlapping 2.7 kb XbaI and 2.1 kb SalI restriction fragments. The two fragments were subcloned from lambda CGN1-2 DNA into pCGN789 (a pUC based vector the same as pUC119 with the normal polylinker replaced by the synthetic linker - 5' GGAATTCGTCGACAGATCTCTGAGCTCGAGGGATCCAAGCTT 3' (which represents the polylinker EcoRI, SalI, BglII, PstI, XbaI, BamHI, HindIII). The identity of the subclones as napin was confirmed by sequencing. The entire coding region sequence as well as extensive 5' upstream and 3' downstream sequences were determined (Figure 2). The lambda CGN1-2 napin gene is that encoding the mRNA corresponding to the BE5 cDNA as determined by the exact match of their nucleotide sequences.

55 An expression cassette was constructed from the 5'-end and the 3'-end of the lambda CGN1-2 napin gene as follows in an analogous manner to the construction of pCGN944. The majority of the napin coding region of pCGN940 was deleted by digestion with SalI and religation to form pCGN1800. Single-stranded DNA from pCGN1800 was used in an *in vitro* mutagenesis reaction (Adelman *et al.*, DNA (1983) 2:183-193) using the synthetic oligonucleotide 5' GCTTGTTGCCATGGATATCTCTGTATGTTTC 3'. This oligonucleotide inserted an EcoRV and an NcoI restriction site at the junction of the promoter region and the ATG start codon of the napin gene. An appropriate mutant was identified by hybridization to the oligonucleotide used for the mutagenesis and sequence analysis and named pCGN1801.

60 65 65 A 1.7 kb promoter fragment was subcloned from pCGN1801 by partial digestion with EcoRV and ligation to

pCGN786 (a pCGN566 chloramphenicol based vector with the synthetic linker described above in place of the normal polylinker) cut with EcoRI and blunted by fill # # resulting expression cassette, pCGN1803 contains 1.725 kb of napin promoter sequence, and 1.265 kb of napin 3' sequences with the unique cloning sites Sall, Bgl, Pst, and Xhol in between. Any sequence that requires seed-specific transcription or expression in Brassica, i.e., a fatty acid gene could be inserted in this cassette in a manner analogous to that described for 5
spinach leaf ACP and the B. napus napin cassette in Example I.

Example III

Other seed-specific promoters may be isolated from genes encoding proteins involved in seed triacylglycerol synthesis, such as acyl carrier protein from Brassica seeds. Immature seed were collected from Brassica campestris cv. "R-500," a self-compatible variety of turnip rape. Whole seeds were collected at stages corresponding approximately to 14 to 28 days after flowering. RNA isolation and preparation of a cDNA bank was as described above for the isolation of a spinach ACP cDNA clone except that the vector used was pCGN565. To probe the cDNA bank, the oligonucleotide (5')-ACTTTCTCAACTGTCTGGTTAGCAGC-(3') was synthesized using an Applied Biosystems DNA Synthesizer, model 380A, according to manufacturer's recommendations. This synthetic DNA molecule will hybridize at low stringencies to DNA or RNA sequences coding for the amino acid sequence (ala-ala-lys-pro-glu-thr-val-glu-lys-val). This amino acid sequence has been reported for ACP isolated from seeds of Brassica napus (Slabas et al., 7th International Symposium of the Structure and Function of Plant Lipids, University of California, Davis, CA, 1986); ACP from B. campestris seed is highly homologous. Approximately 2200 different cDNA clones were analyzed using a colony hybridization technique (Taub and Thompson, Anal. Biochem. (1982) 126:222-230) and hybridization conditions corresponding to Wood et al., (Proc. Natl. Acad. Sci. (1985) 82:1585-1588). DNA sequence analysis of two cDNA clones showing obvious hybridization to the oligonucleotide probe indicated that one, designated pCGN1Bcs, indeed coded for an ACP-precursor protein by the considerable homology of the encoded amino acid sequence with ACP proteins described from Brassica napus (Slabas et al., 1980 supra). Similarly to Example II, the ACP cDNA clone can be used to isolate a genomic clone from which an expression cassette can be fashioned in a manner directly analogous to the B. campestris napin cassette. 10
15
20
25

Other Examples

Ninety-six clones from the 14-28 day post-anthesis B. campestris seed cDNA library (described in the previous example) were screened by dot blot hybridization of miniprep DNA on Gene Screen Plus nylon filters. Probes used were radioactively labeled first-strand synthesis cDNAs made from the day 14-28 post-anthesis mRNA or from B. campestris leaf mRNA. Clones which hybridized strongly to seed cDNA and little or not at all to leaf cDNA were catalogued. A number of clones were identified as representing the seed storage protein napin by cross-hybridization with an Xhol/Sall fragment of pNI (Crouch et al., 1983, supra), a B. campestris genomic clone as a source of an embryo-specific promoter. 30
35

Other seed-specific genes may also serve as useful sources of promoters. cDNA clones of cruciferin, the other major seed storage protein of B. napus, have been identified (Simon et al., 1985, supra) and could be used to screen a genomic library for promoters. 40

Without knowing their specific functions, yet other cDNA clones can be classified as to their level of expression in seed tissues, their timing of expression (i.e., when post-anthesis they are expressed) and their approximate representation (copy number) in the B. campestris genome. Clones fitting the criteria necessary for expressing genes related to fatty acid synthesis or other seed functions can be used to screen a genomic library for genomic clones which contain the 5' and 3' regulatory regions necessary for expression. The non-coding regulatory regions can be manipulated to make a tissue-specific expression cassette in the general manner described for the napin genes in previous examples. 45

One example of a cDNA clone is EA9. It is highly expressed in seeds and not leaves from B. campestris. It represents a highly abundant mRNA as shown by cross-hybridization of seven other cDNAs from the library by dot blot hybridization. Northern blot analysis of mRNA isolated from day 14 seed, and day 21 and 28 post-anthesis embryos using a 700 bp EcoRI fragment of EA9 as a probe shows that EA9 is highly expressed at day 14 and expressed at a much lower level at day 21 and day 28. The restriction map of EA9 was determined and the clone sequenced. Identification of a polyadenylation signal and of polyA tails at the 3'-end of EA9 confirms the orientation of the cDNA clone and the direction of transcription of the mRNA. The partial sequence provided here for clone EA9 (Figure 3) can be used to synthesize a probe which will identify a unique class of Brassica seed-specific promoters. 50
55
60
65

It is evident from the above results, that transcription or expression can be obtained specifically in seeds, so as to permit the modulation of phenotype or change in properties of a product of seed, particularly of the embryo. It is found that one can use transcriptional initiation regions associated with the transcription of sequences in seeds in conjunction with sequences other than the normal sequence to produce endogenous or exogenous proteins or modulate the transcription or expression of nucleic acid sequences. In this manner, seeds can be used to produce novel products, to provide for improved protein compositions, to modify the distribution of fatty acid, and the like. 60

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was 65

specifically and individually indicated to be incorporated by reference.

5 **Claims**

1. A seed comprising an expression cassette, said cassette comprising a seed specific transcriptional initiation region, a sequence of interest under the transcriptional regulation of said initiation region, and a transcriptional termination region, said expression cassette inserted into the genome of said seed at other than the natural site for said transcriptional initiation region.
- 10 2. A seed according to Claim 1, wherein said sequence of interest is an open reading frame encoding an endogenous protein or mutant thereof.
- 15 3. A seed according to Claim 1, wherein said sequence of interest is an open reading frame encoding an exogenous protein.
4. A seed according to Claim 1, wherein said sequence of interest encodes a sequence complementary to a transcription product of said seed.
5. A seed according to Claim 1, wherein said seed is of the Brassica family.
- 20 6. An expression construct comprising a seed specific transcriptional initiation region, a polylinker of less than about 100 bp having at least two restriction sites for insertion of a DNA sequence to be under the transcriptional control of said initiation region, and a transcriptional termination region, the sequence of said polylinker being other than the sequence of the gene naturally under the transcriptional control of said initiation region.
7. An expression construct according to Claim 6, wherein said transcription initiation region is from a Brassica seed gene.
- 25 8. An expression construct according to Claim 7, wherein said gene is a napin gene.
9. An expression cassette comprising a seed specific transcriptional initiation region, a DNA sequence of interest, other than the natural sequence joined to said initiation region, to be under the transcriptional control of said initiation region, and a transcriptional termination region.
- 30 10. An expression cassette according to Claim 9, wherein said transcriptional initiation region is a Brassica gene initiation region.
11. An expression cassette according to Claim 10, wherein said gene is a napin gene.
12. An expression cassette according to Claim 10, wherein said sequence of interest is a structural gene.
13. An expression cassette according to Claim 12, wherein said structural gene encodes a protein in the biosynthetic pathway for fatty acid production.
- 35 14. An expression cassette according to Claim 13, wherein said protein is acyl carrier protein.
15. A vector comprising an expression cassette according to any of Claims 9 to 14, a prokaryotic replication system, and a marker for selection of transformed prokaryotes comprising said marker.
16. A method for modifying the genotype of a seed comprising:
growing a plant to seed production, wherein cells of said plant comprise an expression cassette according to any of Claims 9 to 14,
whereby seeds are produced of modified genotype.

45

50

55

60

65

0255378

NAP-270 Linear

LENGTH = 702

1 AAAGGGATGTGTCTGGTATGTATCTACGAGTAACAAAAGAGAAGATGCAATTGAGTAGTAGAA5GATTEG
19 31 35
36

AluI AhaIII FokI EcoRV HgaI
72 AGAGCTTT45AAGCC34TCA5GTGTGCTT4ATCTTATTGATATCATCCATT4GCGTGTGTTAATGCG 1
76 82 106 117 130

DdeI
143 TCTTAGATATGTTCTGTTCTTCTCAGTGTCTGAATATCTGATAAGTCAATGTGAGAAAGCCACACC 2
169

SspI TaqI HinfI
214 AACCAAAATATTCAAATCTTATATTTAATAATGTCGAATCACTCGGAGTTGCCACCTCTGTGCCAAT 2
224 253 251

HinfI MboII EcoRI
285 TGTGCTGAATCTATCACACTAAAAAAACATTCTCAAGGTAATGACTTGTGGACTATCGTTCTGAATTC 3
292 310 351

MaeII
356 TCATTAAGTTTATTTTGAAAGTTAAGTTTACCTTCTTTGAAAAATATCGTTCATAGATGTC 4
424

ScrFI NlaIII SphI Nsp(7524)I MaeIII
EcoRII AluI Sfani NlaIII
427 ACGCCAGGACATGAGCTACACATCACATATTAGCATGCAGATGCGGACGATTGTCACTCACTCAAACAC 4
430 442 457 464 480
432 440 464
464

Tth111II		SspI	PmaCI
AluI		SphI	MboI
		NdeI	Nsp(7524)I
498 CTAAAAGAGCTTCTCTCACAGCACACACACATATGCATGCAATATTACACGTGATGCCATGCAAATC		MaeII	
507	532	539	548
508	531	539	555
			563
		539	553
		543	551

HphI	MnlI
569 TCCATTCTCACCTATAAATTAGAGGCTCGGCTTCACTTTACTCAAACCAAAACTCATCACTACAAACA	6
569	583

MboII	AluI	MnlI
640 TACACAA <u>ATGGCGAACAAAGCTTCC</u> TCGTCTGGCAACTCTGCCTGTTCTCCTTCAC	702	
653	659	675

SITE [NAP-270]: Q

Partial sequence of the promoter region of the λ BnNa napin gene. The start (ATG) of the open reading frame is underlined. Unconfirmed or ambiguous nucleotides in the sequence are designated by numbers.

0255378

Lambda CGN1-2

NCG-186 Linear

LENGTH = 4325

XbaI

TaqI

Avai

HindIII

AluI

TaqI

1 CTCGAGGCAGTCACTAACATGAAGTTGACGAGGAGCCAACTATGGGAAGCTTATTCCTTTCCAT 69

2

3

2

52

66

50

Bhai XbaI

SacI

AluI

70 ACTCTAATTGAGCCGTGCGCTCTATCTAGACCAATTAGAATTGATGGAGCTCTAAAGGTTGGCTGT 138

89 95

119

121

NdeI

NdeI

139 TTTCTTGTTCATATGATTAACCTCTAACTTGTGTATAAAATATTCTCTGAAAGTGCCTCTGGCATA 207

150

206

208 TGTAGGTTGGCAAAACGAGGAAGATGCTTCTCAATTGGAAGAGGATGAAACAGCCGAAGAAGAAA 276

Sau3AI

DdeI

277 TAAGAATAGGCAGTCCTGCTACTCAATGGATCTCAGTCTATAACGGTCGTCCCCTGAAACAGAGGT 345

309

305

EcoRV

346 AAAACATTTTGCATATACACTTTGAAAGTCCTCACTAACTGTGTAATCTTTGGTAGATATCACTA 414

408

HincII
HhaI
HaeIII
DdeI
BstEII
Bali
|||
HaeIII AluI

415 CAATGTCGGAGAGACAA3GGCTGMNCANCATATACAAAAGGGAAATGAAGATGGCCTTTGATTAGCTG 483

439 469 481
438
439
439
440
438

AluI

HincII

484 TGTAGCATCAGCAGCTAATCTCTGGGCTCTCATCATGGATGCTGGAAGTGGATTCACTTCTCAAGTTA 552

498

535

MspI
HpaII

HincII

553 TGAGTTGTACCGGTCTCCTACACAAAGGTAAATAATCAGTTGAAGCAATTAAAGATCAATTGATTGT 621

564
564

606

DdeI

622 AGTAAGCTAAGAAGAACTTACCTTATGTTTCCCCCGCAGGACTGGATTATGGAACAATGGAAAAGAAC 690

629

AluI AluI

SacI

AluI

691 TACTATATAAGCTCCATAGCTGGTTCAAGATAACGGGAGCTTTAGTTGTTATGTCAAAAGGTTAGTGT 759

702 710

729

731

760 TTAGTGAATAATAACTTATACACAAAGTCTTCATTGACTTATTTATATACTTGTGTGAATTGCTAG 828

DdeI

HincII

829 GAACTACTTATTCTCAGCAGTCATACAAAGTGAGTGACTCAATTCCGTTCAAGTGGATAAAATAAGAAAT 897

842

865

0255378

XmnI

TaqI

898 GGAAAGAAGATTTCATGTAACCTCCATGACAAC TGCTGGTAATCGTTGGGTGTGGTAATGTCGAGGA 966
908 961

Sau3AI
BclI

967 ACTCTGGCTTCTCTGATCAGGTAGGTTTGTCCTTATTGTCGGTGTGGTACCTTCCCCTGAGT 1035
981 981

AluI

RsaI

1036 CTAATATGATAAACTCTGCCTGTGAAAGGTGGTGGAGCTTGACTTTTGTAACCAAGCGATGGGATAC 1104
1074 1087

Sau3AI AluI

1105 ATAGGAGGTGGGAGAATGGGTATAGAATAACATCAATGGCAGCAACTGCGGATCAAGCAGCTTCATAT 1173
1155 1165

HinfI

ScaI

RsaI

1174 TAAGCATAACCAAGCGTAAGATGGTGGATGAAACTCAAGAGACTCTCCGCACCACCGCCTTCCAAGTA 1242
1215 1242
1242

AluI

Sau3AI

DdeI

1243 CTCATGTCAAGGTTGGTTCTTAGCTTGAAACACAGATTGGATCTTTGTTTGTTCATATACT 1311
1268 1285 1311

DdeI

Avall AluI

HinfI RsaI

1312 TAGGACCTGAGAGCTTTGGTGATTTTTTCAGGACAAATGGCGAAGAATCTGTACATTGCATCA 1380
1315 1325 1363 1370
1319

1381 ATATGCTATGGCAGGACAGTGTGCTGATAACACACTTAAGCATCATGTGGAAAGCCAAAGACAATTGGAG 1449

0255378

HinfI
DdeI

1450 CGAGACTCAGGGTCGTATAATACCAATCAAAGACGTAAAACCAGACGCAACCTCTTGGTTGAATGTA 1518

1456
1454

RsaI

1519 ATGAAAGGGATGTGTCTTGGTATGTATGTACGAATAACAAAAGAGAAGATGGAATTAGTAGTAGAAATA 1587

1548

AluI

EcoRV

1588 TTTGGGAGCTTTTAAGCCCTCAAGTGTGCTTTATCTTATTGATATCATCCATTGCGTTGTTAA 1656

1596

1635

XbaI

DdeI

1657 TGCCTCTAGATATGTTCTATATCTTCTCAGTGTCTGATAAGTGAATGTGAGAAAACCATAACAA 1725

1664

1687

HinfI

1726 ACCAAAATATTCAAATCTTATTTAATAATGTTGAATCACTCGGAGTTGCCACCTCTGTGCCAATTG 1794

1761

HinfI

EcoRI

1795 TGCTGAATCTATCACACTAGAAAAAAACATTCTCAAGGTAATGACTTGTGGACTATGTTCTGAATTC 1863

1800

1859

1864 TCATTAAGTTTATTTCTGAAGTTAAGTTTACCTTCTGTTGAAATATATCGTTCATAGATG 1932

SphI
Sau3AI

BstNI AluI

1933 TCACGCCAGGACATGAGCTACACATCGCACATAGCATGCAGATCAGGACGATTGTCACTCACTCAAA 2001

1940 1950

1973
1971

DdeI	AluI			SphI		
HhaI		NdeI	NsiI			Sau3AI
2002	CACCTAAGAGCTTCTCTCACAGCGCACACACATATGCATGCAATATTACACGTGATGCCATGCAA	2070				
2006	2012	2028	2036	2042	2058	
				2044		

2071 ATCTCCATTCTCACCTATAAATTAGAGCCTCGGCTTCACTCTTACTGAAACCAAACACTCATCACTACA 2139

	AluI					
2140	GAACATACACAAATGGCGAACAAAGCTTCCTCGTCTCGGCAACTCTCGCCTTGTCTTCCTTCACG	2208				
	METAlaAsnLysLeuPheLeuValSerAlaThrLeuAlaLeuPhePheLeuLeuThr					
	2164					

	TaqI			NaeI		
	Sali			MspI		
	HincII			HpaII		
AccI	AccI	AccI		HaeIII		
2209	AATGCCCTCGTCTACAGGACGGTTGTGGAAGTCGACGAAGATGATGCCACAAATCCAGCCGGGCCATT	2277				
	AsnAlaSerValTyrArgThrValValGluValAspGluAspAspAlaThrAsnProAlaGlyProPhe					
2220	2241	2241		2271		
	2239	2240		2268		
	2241			2268		
				2269		

	HinfI			HindIII		
				AluI		
2278	AGGATTCCAAAATGTAGGAAGGAGTTTCAGCAAGCACACACCTGAAAGCTTGCCAAACAATGGCTCCAC	2346				
	ArgIleProLysCysArgLysGluPheGlnGlnAlaGlnHisLeuLysAlaCysGlnGlnTrpLeuHis					
2281	2327	2325				

	MspI			AvaII		
	HpaII		AluI	TaqI		
2347	AAGCAGGCAATGCAGTCCGGTAGTGGTCCAGCAGCTGGACCCCTCGATGGTGAGTTGATTTGAAGACGAC	2415				
	LysGlnAlaMETGlnSerGlySerGlyProSerTrpThrLeuAspGlyGluPheAspPheGluAspAsp					
2364	2372	2379	2388	2382		

	HaeIII		SacI	
	Apal	HaeIII	AluI	
2416	GTGGAGAACCAACAAACAGGGCCCGCAGCAGAGGCCACCGCTGCTCCAGCAGTGCTGCAACGAGCTCCAC	2484		
	ValGluAsnGlnGlnGlnGlyProGlnGlnArgProProLeuLeuGlnGlnCysCysAsnGluLeuHis			
	2438	2449	2479	
	2436		2481	
	BstNI		TaqI	
			HinfI	
2485	CAGGAAGAGCCACTTGCCTTGCCAACCTTGAAAGGAGCATCCRAAGCCGTTAACAAACAGATTGCA	2553		
	GlnGluGluProLeuCysValCysProThrLeuLysGlyAlaSerLysAlaValLysGlnGlnIleArg			
	2486		2548	
			2551	
2554	CAACAAACAGGGACAACAAATGCAGGGACAGCAGATGCAGCAAGTGATTAGCCGTATCTACCAGACCGCT	2622		
	GlnGlnGlnGlyGlnGlnMETGlnGlyGlnGlnMETGlnGlnValIleSerArgIleTyrGlnThrAla			
	AluI		BstNI	
2623	ACGCACTTACCTAGAGCTTGCAACATCAGGCAAGTAGCATTGCCCCCTCCAGAAGACCATGCCCTGGG	2691		
	ThrHisLeuProArgAlaCysAsnIleArgGlnValSerIleCysProPheGlnLysThrMETProGly			
	2639		2689	
	MspI			
	HpaII			
	HaeIII	XbaI		
	Apal	TaqI		
	HinfI	Avai	AccI	
2692	CCCGGCTTCTACTAGATTCAAACGAATATCCTCGAGAGTGTGTATACCACGGTATATGAGTGTGGTT	2760		
	ProGlyPheTyr			
	2694	2707	2724	2736
	2692		2725	
	2694		2724	
	2694			
	HincII		RsaI	
2761	GTTGATGTATGTTAACACTACATAGTCATGGTGTGTTCCATAAATAATGTAATGTAATAAGAAC	2829		
	2771		2813	
	AccI			
2830	TACTCCGTAGACGGTAATAAGAGAGAAGTTTTTTACTCTTGCTACTTCCATAAAAGTGTGATGAT	2898		
	2838			

Scal
RsaI

2899 TAACAAACAGATAACACCAAAAAGAAAACAATTAATCTATATTACAATGAAGCAGTACTAGTCATTGAA 2967

2954
2954

Sau3AI

2968 CATGTCAGATTTCTTTCTAAATGTCTAATTAAGCCTCAAGGCTAGGATGATAAAAGATCATCCA 3036

3028

Sau3AI
BamHI

HinfI

Sau3AI
BclI

3037 ATGGGATCCAACAAAGACTCAAATCTGGTTTGATCAGATACTTCAAAACATTTTGTATTCACTAAA 3105

3041
3041

3053

3069
3069

HinfI

3106 TTATGCAAGTGTCTTTATGGTGAAGACTCTTAGAAGCAAAGAACGACAAGCAGTAATAAAAAAA 3174

3135

3175 ACAAAAGTTCAAGTTTAAGATTGTTATTGACTTATTGTCATTGAAAAATATAGTATGATATTAATATA 3243

3244 GTTTTATTATATAATGCTTGTCTATTCAAGATTGAGAACATTAATATGATACTGTCCACATATCCAA 3312

NdeI

3313 TATATTAAGTTCAATTCTGTCATATGATAAGATGGTCAAATGATTATGAGTTTGTATTAC 3381

3341

TaqI
AluI

Sau3AI

RsaI

3382 CTGAAGAAAAGATAAGTGAGCTCGAGTTCTGAAGGGTACGTGATCTCATTCTGGCTAAAGCGA 3450

3402

3405

3421

3425

3451 ATATGACATCACCTAGAGAAAGCCGATAATAGTAAACTCTGTTCTGGTTTGGTTAACTCAACCGA 3519

$ \begin{array}{l} \text{MspI} \quad \text{DdeI} \\ \quad \\ \text{HpaII} \quad \text{AluI} \end{array} $	$ \begin{array}{l} \text{NdeI} \\ \\ \text{HinfI} \end{array} $	$ \begin{array}{l} \text{MspI} \\ \\ \text{HpaII} \end{array} $
3520 ACCGGTAGCTGAGTGTCAAGTCAGCAAAACATCGCAAACCATATGTCAATTGTTAGATTCCCGTTAA 3588		
$ \begin{array}{l} 3522 \quad 3528 \\ 3522 \quad 3529 \end{array} $	$ \begin{array}{l} 3560 \end{array} $	$ \begin{array}{l} 3576 \\ 3581 \\ 3581 \end{array} $
$ \begin{array}{l} \text{MspI} \\ \\ \text{HpaII} \end{array} $	$ \begin{array}{l} \text{HinfI} \\ \\ \text{HincII} \end{array} $	$ \begin{array}{l} \text{BstNI} \\ \\ \text{DdeI} \end{array} $
3589 GTTGTAAACCGGTATTCATTGGTAAAAACCTAGAACGCCAGCCANCCTTTAACTAATTTGCA 3657		
$ \begin{array}{l} 3598 \\ 3598 \end{array} $	$ \begin{array}{l} 3702 \end{array} $	$ \begin{array}{l} 3718 \\ 3715 \\ 3714 \end{array} $
3658 AACGAGAAGTCACCACACCTCTCCACTAAAACCTGAAACCTTACTGAGAGAAGCAGAGNCANAAAGAA 3726		
$ \begin{array}{l} \text{AvaiI} \\ \\ \text{AluI} \end{array} $	$ \begin{array}{l} \text{AvaiI} \\ \\ \text{DdeI} \end{array} $	$ \begin{array}{l} 3804 \\ 3801 \end{array} $
3796 CGGC GG5MNTTGGTGGCGGCGGCGGACGTTTGGTGGCGGCGGTGGACGTTTGGTGGCGGCGGTGGA 3864		
$ \begin{array}{l} \\ \\ \text{EcoRV} \end{array} $	$ \begin{array}{l} \\ \\ \text{AvaiI} \end{array} $	$ \begin{array}{l} \\ \\ \text{DdeI} \end{array} $
3865 CCTTG GTGGATATCGT GACGAAGGACCTCCAGTGAAGTCATTGGTTGTTACTCTTTCTTAG 3933		
$ \begin{array}{l} 3880 \end{array} $	$ \begin{array}{l} 3892 \end{array} $	$ \begin{array}{l} 3930 \end{array} $

TaqI | HindIII
 HinfI | AluI | DdeI
 3934 TCGAATCTTATTCTTGCCTGCTCGTTACCGATAAAGCTTAAGACTTATTGATAAAGTTCTCA 4002
 3937 3976 4000
 3935 3974

 AluI | XbaI | HinfI | DdeI
 4003 GCTTTGAATGTGAATGAACTGTTCCCTGCTTATTAGTGTTCCTTGTGAGTTGAATCACTGCTTA 4071
 4004 4023 4059 4069

 HinfI |
 4072 GCACTTTGTTAGATTCATCTTGTGTTAAGTTAAAGGTAGAAACTTGTGACTTGTCTCCGTTATG 4140
 4085

 KincII |
 4141 ACAAGGTTAACCTTGTGGTTATAACAGAAGTTGCGACCTTCTCCATGCTTGTGAGGGTGATGCTGTG 4209
 4146

 AvaiI | AluI | DdeI | Sau3AI
 4210 GACCAAGCTCTCAGCGAAGATCCCTTACTTCAATGCCCAATCTACTTGGAAAACAAGACACAGAT 4278
 4210 4217 4222 4231

 TaqI |
 SalI |
 PstI |
 HindIII | HincII |
 Sau3AI | AluI | AccI | EcoRI |
 4279 TGGGAAAGTTGATGAGATCCAAGCTGGGCTGCAGGTCGACGAATTC 4325
 4294 4302 4316 4321
 4300 4314
 4313
 4315
 4316

EA9-14 Linear

LENGTH = 416

ScrFI

MaeIII

HpaII

CauII

MnII

SphI

Nsp

1 CCAACCCGGTTACAATTGGAAATAGCGGGAGGAGATATTGGTTCCATTCTATTCTCGTCCGGTGTGTCAGC

7

21

59

71

7

9
7

XbaIII

MboI

HaeIII

DpnI

BsmI

BclI

MaeIII

GdIII

CfrI DpnI

72 GGAGAAATGCACAACTCATCTGATCACGGGGTAAGTGCAGGGTGGATACGGCCGATCTAAAAACGGATTAAA 145

82

93

102

120 126

95

120

93

122

93

124

120

XbaII

ScaI NlaIV

NlaIV

NlaIII

MboI

FniI

MboI

RsaI DpnI

EcoRI

AvaiI

MnII

BlnI BamHI

BlnI

MnII

MaeII

MboI

143 GTACTGGATCCTCAAGAATTCAATGGGGACCAAAATGGGGAGAACGTGATACTAGGAGTCAAAAAGATA 213

144 149

157

163

169

186

202

208

213

145 151

159

169

190

200

149

167

167

198

145 151

167

167

149

170

NlaIII RsaI

BsmI

214 TCAAGCCTAACACGGACAAATGTGGTCTGCCATGAATGCTTCGTACCCAACTATGTGAAAAAATCGGTT 284

253

249 259

HindIII

HpaII

AluI

Nsp(7524)I

NlaIII

285 AATATCCGGTTAAGCTTACAATAATGTGTGTGGTTATAATTAAAGACTCTGTTGCATGTAAATTGT 355

291

299

335

348

297

348

0255378

Sfani

356 GAAATGGTAAGTTATGTGATGCAAAAAGATTTGATAAAAAAAAAAGGGGGGGGGGG 416
365

Partial sequence of B. campestris seed cDNA 2A9 sufficient to identify a genomic clone containing a promoter. The polyadenylation signal AATAAA is underlined as are the polyA tails. The stop codon of the presumed open reading frame is double underlined.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER: _____**

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.